THE USE OF DECOLORIZED ACID FUCHSIN AS AN ACID INDICATOR IN CARBOHYDRATE FERMENTATION TESTS WITH SOME REMARKS ON ACID PRODUCTION BY BACTERIA *

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In 1895 Andrade-Penny[1] reported his results on the use of "acid fuchsin for the differentiation of bacteria." This author employed an aqueous solution of acid fuchsin (fuchsin S. Grubler) and found it to be an excellent indicator for acids and alkalies. When added to various glycerin media it served to demonstrate the reactions which resulted from the growth and activity of certain of the intestinal bacteria.

In 1906, Andrade[2] carried the work further, and he strongly advocated the use of glycerin, Dunham's solution, with the acid fuchsin indicator for the differentiation of the bacillus typhosus. For some time our laboratory was employing this medium as a routine for the purpose suggested, and it occurred to us that the indicator could be extended to the other carbohydrates used in our fermentation tests. I therefore added it to various carbohydrate media, and the results proved it to be a very useful addition to our bacteriological technic. I have found it eminently satisfactory as an indicator of the production of acid resulting from the fermentation of carbohydrates by bacteria, and realizing that it is employed in very few bacteriological laboratories, I feel that its use should be more widely extended.

The indicator is prepared as follows:

Acid fuchsin (fuchsin S. Grubler)	0.5 gm.
Distilled water	100 c.c.

To this solution, which is of rich magenta color, normal sodium hydrate is added until the color is changed to pink, then to a brownish red and this, in turn, is changed to yellow. The change of color takes place slowly, and the solution must be thoroughly shaken after each addition of the alkali. Andrade remarked that a pale pink solution

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1. *Ann. Rep. U. S. M.-H. S.*, 1895, p. 385.
2. *Jour. Med. Research*, 1906, 14, p. 551.

will become yellow after standing for an hour or two. It is most practical to add the normal sodium hydrate until a definite yellow color is obtained. It usually takes about 17 c.c. normal soda solution to completely decolorize 100 c.c. of the 0.5 per cent. fuchsin solution. One c.c. of this indicator is added to 100 c.c. of the carbohydrate medium.

I have found that broth made + 0.6 to phenolphthalein (hot titration) remains colorless on the addition of the fuchsin indicator prepared as described above. During sterilization at 100 C. the broth turns a distinct pink. This color disappears on cooling, and the medium remains colorless at room and incubator temperature.

Undoubtedly the acid in the broth is partially neutralized by the addition of the indicator, as the final broth is found to be neutral to phenolphthalein in the cold. I have found as has Winslow[3] and others that the use of phenolphthalein at 100 C. shows slightly higher acid reaction than when used in the cold. This gives a double control on a neutral point for the broth; a slight reaction in the cold to phenolphthalein but not to the fuchsin indicator, and at 100 C. a slight acid reaction to the fuchsin but none to phenolphthalein.

The indicator is made up in large quantities. It should be tested twenty-four hours after the last addition of the alkali by adding it to + 0.6 broth in 1 per cent. quantities. A pink color appears on boiling and this on cooling fades, leaving the broth unchanged in color.

This indicator is very sensitive to the presence of the organic and mineral acids. One thousand c.c. of water containing 1 drop of concentrated lactic acid, butyric acid, acetic acid, or tannic acid (20 per cent. solution) turns pink on the addition of the indicator.

During the fermentation of the various carbohydrates by bacterial growth, organic acids (most frequently lactic acid) are produced, any one of which, acting on the fuchsin salt, sets free the fuchsin, and turns our media pink or red. The depth of the color depends roughly on the amount of acid produced.

The fuchsin salt is very stable. It is not affected by heating to 100 C. and over, and no dissociation takes place after the indicator has remained for years in the media. It has no appreciable effect on the growth of even the most sensitive organisms.

Mereshkowsky[4] says that fuchsin in a dilution of 1-1,000 delays, to some extent, the action of invertase, and occasionally renders it

3. Systematic Relationships of the Coccaceae, New York, 1908.
4. Quoted by Fuhrmann, Bakterienenzyme, Jena, 1907.

inactive. The fuchsin, as used in the Andrade indicator, is in a dilution of over 1-20,000 and in our experience has no demonstrable effect in checking or delaying the action of this ferment in the fermentation of saccharose. I have made a number of comparisons to definitely determine this point, using azolitmin and Andrade's fuchsin as indicators. A quantity of saccharose broth was divided into equal parts, the two indicators added, tubed in Durham's fermentation tubes, and sterilized together. A number of strains of the bacilli proteus, clocae, coli communior, lactis aërogenes, acidi lactici, xerosis, together with yeast were used to seed the medium. The results showed: (1) that acid was indicated earlier in the tubes containing Andrade's indicator; (2) that no acid was shown by either indicator in the tubes seeded with non-saccharose fermenting bacteria; (3) that the amount of gas formed by the acid and gas fermenters was the same; and (4) that the litmus was rapidly decolorized by many of the strains making the reading for acid difficult or impossible. From these results and experience extending over several years, I conclude that Andrade's indicator has decided advantages over litmus or azolitmin in that: the indication of the presence of acid is clear-cut and definite; the cultures may be examined and the reading made as well by artificial light as by daylight; it is unnecessary to make comparisons with a control tube; as our media in the neutral state is colorless, there is not the difficulty of making every batch of media exactly the same shade of violet as with litmus; litmus is also much more easily decolorized by reduction, which adds to the difficulty of determining acid production.

For teaching purposes and for use by the student, Andrade's indicator fills a long-felt want.

The method of titrating with phenolphthalein, in order to obtain the exact percentage of acid produced after different periods of growth, is too cumbersome for routine laboratory work and offers little or no help in the diagnosing of our cultures. In the differentiation of bovine from human strains of the bacillus tuberculosis, however, the titration determinations are of great importance as first shown by Theobald Smith. There are, however, too many variable factors to be considered to make it of practical use excepting in these very special biological researches. The number of organisms transferred, the age of the culture used for the planting, and the late previous environment of the strain, all lead to wide differences in the exact percentage of acid pro-

duced in a given time by one and the same organism. It is often difficult, when one makes transfers of one organism to a number of different tubes or flasks of even the same lot of medium, to obtain the same titration results from the whole series. Working with different lots of media, however carefully made, the difficulty of obtaining identical results is increased.

Andrade's indicator is useful in making titration determinations of the acid produced, where this is particularly desired. Measured quantities of alkali are added to known amounts of media in test tubes or flasks until the pink color disappears. Precaution must be taken to allow sufficient time for the adjustment between the acid and alkali to take place.

The presence of slight amounts of alkali in carbohydrate media does not interfere with the indication of acid production as so many investigators seem to believe. Unless the alkali is present in such quantities as actually to prevent the growth of the organisms, the acid soon neutralizes the alkali and growth and fermentation continues until the acid produced is sufficient to either kill the bacteria or to interfere with their specific biological functions. Winslow in a study of thirty-three strains of cocci concludes that an excess of acid over 1 per cent. is more generally fatal than an alkaline reaction. The presence of acid in media has a very detrimental effect on the life of many bacteria, such as the cholera vibrio, certain streptococci, and many others.[5] The addition of carbohydrates to media increases the growth of bacteria, which have the power to ferment the particular carbohydrate, but lessens the longevity of the organisms.

The acid produced in fermentation first affects the biological function of producing ferments, or lessens the action of such ferments if produced, and then the acid gradually kills the bacteria. It is true, however, that very rarely ferments have been known to outlive the bacteria.[6] Fuhrmann[7] refers to the large number of bacteria which break up the simple sugars to lactic acid, and says that these bacteria are very sensitive to the action of acids, especially lactic acid, which checks their growth and activity.

The fermentation of a given carbohydrate by an organism is a definite biological character of that organism. The acid death point is a question of vital resistance. The acid point at which bacterial

5. Lafar, Handb. d. Tech. Mykol., Jena, 1907; *Am. Jour. Pub. Health,* 1913, 3, p. 1210.
6. Lafar, Handb. d. Tech. Mykol., Jena, 1907.
7. Bakterienenzyme, Jena, 1907.

functions cease is a question of biological functional resistance. I have shown that by adding sterile sodium hydrate solution (N/20) to dextrose broth cultures of streptococci each day, to prevent the accumulation of acid, the total acid produced is increased three to four times over that in the unneutralized controls. Similar methods were used by Fischer[8] in determining the acid production of the bacilli coli and paratyphosus.

The determination of the acid death point and the point at which biological activities cease, or are depressed, should be distinguished from the determination of the qualitative fermentative powers of bacteria.

Biometrical studies of fermentation powers of bacteria, in which an arbitrary acid point is spoken of as fermentation while all acid production below this point is neglected, are of great interest as indicating the variable sensitiveness of different bacteria and their ferments to the acids produced, though of little use in determining whether the organism has or has not the power to reduce the carbohydrates. Winslow[9] for streptococci fixed the fermentation point at + 1.2 to phenolphthalein, while Broadhurst uses + 1.5. Streptococci showing acid reactions at or below these points are spoken of as nonfermenters. A reaction of + 0.8 per cent. turns litmus a decided red.[10] What is the source of their + 1.2 or + 1.5 per cent.? I believe it to be the result of the fermentation of the contained carbohydrate. The biometrical system of classification by fermentation tests of Andrewes and Horder[11] and the other English workers is based on qualitative changes. It is true, that subdivisions might be made by the use of further quantitative determinations, but much more must first be learned of the sensitiveness of the bacteria and the conditions of the activity of their enzymes before such a classification is, we believe, advisable. We have found as have many investigators that strains of the bacillus coli recently isolated from water show marked depression in their fermentative powers. Henningsson[12] has shown that cultures of the bacillus coli kept in water for long periods will suffer quantitative loss in their fermentative powers. These depressions are not, however, considered worthy of use in classification.

9. Systematic Relationships of the Coccaceae, New York, 1908.
8. *Centralbl. f. Bakteriol.*, 1911, 59, p. 474.
10. *Ibid.*
11. *Lancet*, 1908, 171, p. 708.
12. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, 74, p. 253.

It is well known that different carbohydrates broken up by fermentation give different reduction products. Lactic acid is most constantly found, but oxalic, butyric, acetic, succinic, formic, and other acids are also formed.[13] Moreover, different bacteria break up the same carbohydrate with varying final products.

The fact that bacteria are destroyed by the acids produced in fermentation is well established. It also appears that certain ferments formed by the bacteria are inactive in acid media, or are not formed under the unfavorable acid environment, because the amount of acid produced by the same organism on different carbohydrates varies. Winslow,[14] Fuller and Armstrong,[14] and others, have shown that the amount of acid produced by streptococci from the reduction of dextrose is much greater than that from the more complex carbohydrates, such as lactose, mannite and raffinose. Broadhurst[15] shows that the percentage of acid from salicin is higher than from these three carbohydrates.

The fact that acid is produced by these organisms from the higher carbohydrates, even though the amount is less than from the lower forms, indicates that the power of reducing these carbohydrates is present, and that fermentation does not continue must be due to the presence of the acid products and the lowering of the specific functional activity of the organism or the ferment. Bacterial invertase, for example, is sensitive to acid. Emulsin, however, is not affected by the organic acids (Fuhrmann).[16]

We have used this neutralized acid fuchsin indicator in studying the fermentation of the various carbohydrates; dextrose, maltose, lactose, saccharose, dulcit, raffinose, salicin, mannite, glycerin, dextrin, and others, with a large number of different bacteria including the pneumococcus, streptococcus, the colon-typhoid, and the diphtheria groups, the Gram-negative cocci, and many others, and have found it very satisfactory. The secondary alkali production, among certain of the bacteria, is definitely shown by the decolorization of the indicator. This decolorization is, however, at times, due to the reducing power of the bacteria on the fuchsin. We have noted it particularly among members of the bacillus mucosus capsulatus group. The decoloriza-

13. Gotschlich, Kolle and Wassermann, Handb. d. path. Mikorg., Jena, 1913; Fuhrmann, Bakterienenzyme, Jena, 1907; Lafar, Handb. d. Tech. Mykol., Jena, 1907.
14. Systematic Relationships of the Coccaceae, New York, 1908; *Jour. Infect. Dis.*, 1913, 13, p. 442.
15. *Jour. Infect. Dis.*, 1912, 10, p. 272.
16. Bakterienenzyme, Jena, 1907.

tion, due to the reducing power of the bacteria, appears much less often and at a far later period of growth than when litmus is used.

CONCLUSIONS

The neutralized acid fuchsin indicator as used by Andrade in his glycerin media is applicable for testing the production of acid in the bacterial fermentation of all carbohydrates.

The technic of obtaining a neutral point in media by hot titration with phenolphthalein controlled by the acid fuchsin indicator, showing pink when hot and colorless when cold, gives the most satisfactory results.

Andrade's neutralized acid fuchsin is superior to litmus, as an indicator of acid production in media, on account of its sensitiveness, the sharpness of the change of color (Cohn),[17] and the higher resistance to reduction decolorization. It is particularly useful for teaching purposes.

Titration determinations can be carried out with this indicator at any stage in the growth of the culture.

The use of this indicator in all bacteriological laboratories would give better and more comparable results in studies on the fermentation of the carbohydrates.

Qualitative tests for fermentation are more important than quantitative in the classification of bacteria.

The resistance of bacteria and their ferments to the effects of acid produced in the fermentation process differs with the carbohydrate reduced and with various strains of the same organism. These differences are not, we believe, sufficiently understood to warrant their use in classification.

17. Indicators and Test Papers, New York, 1910, p. 2.